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14. ABSTRACT
Cancer and normal development share many properties. During normal development, genes are activated that stimulate proliferation, migration, invasion, vascularization, and that alter cell survival. These gene products are often lost once organ development is complete. In cancer, many developmental genes are re-activated, stimulating the aforementioned processes out of context. The Six1 gene encodes a transcription factor that induces the expression of a large number of genes that are involved in the proliferation, survival, migration, and invasion of cells during embryonic development. In most tissues, Six1 expression is lost once development is complete. However, Six1 is reactivated in many breast cancers, where as many as 90% of metastatic tumors overexpress the gene. Six1 plays a role in both tumor initiation and metastasis of breast cancers, and its inhibition dramatically diminishes both tumor cell proliferation and metastasis in a number of mouse cancer models. Because Six1 is expressed during embryogenesis, lost in most adult tissues, and re-expressed in tumors, we believe it is an ideal drug target whose inactivation will inhibit tumor cell proliferation and metastasis with limited side effects. Our goal in this proposal is to lay the foundation for developing novel, tumor-specific chemotherapeutic agents for breast cancer. This will be accomplished by coupling the expertise of a cancer/molecular biologist with a structural biologist/biochemist. Within the proposal, we will identify multiple avenues for targeting the Six1 transcriptional complex, and use an innovative rational drug design and complementary high throughput screeing (HTS) approach to identify small molecule inhibitors of the Six1 complex. The Six1 transcriptional complex has never before been clinically targeted- but its inhibition would be expected to inhibit both tumor cell proliferation and metastasis, while sparing normal cells. Such a target is badly needed in breast cancer, where many of the currently used therapies have serious side effects. This research is expected to benefit 50% of breast cancer patients with primary breast tumors and 90% with metastatic tumors, as Six1 is expressed in the aforementioned percentage of breast cancer cases. We project to have lead compounds targeting Six1 within five years. The DOD Synergistic IDEA grant will greatly facilitate our efforts in generating lead compounds as soon as possible.

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INTRODUCTION

In this proposal we are performing structural and functional analyses of the Six1 transcriptional complex for anti-breast cancer drug design. Six1 is a transcription factor that has never before been clinically targeted and that plays a critical role in the onset and progression of a significant proportion of breast cancers. Six1 expression is low or undetectable in normal breast tissue but the gene is overexpressed in 50% of primary breast tumors and 90% of metastatic lesions. Furthermore, examination of public microarray databases containing more than 580 breast cancer samples demonstrates that it correlates significantly with shortened time to relapse, shortened time to metastasis, and decreased overall survival. Using mouse models of mammary cancer, we have demonstrated that its overexpression results in enhanced proliferation, transformation, increased tumor volume, *and* metastasis. Importantly, RNA interference against Six1 decreases cancer cell proliferation and metastases in several different cancer models. The Eya proteins are co-activators of Six1 that utilize their intrinsic phosphatase activity to switch the Six1 transcriptional complex from a repressor to an activator complex. The Six1-Eya interaction is essential for proliferation during embryonic development, and both Six1 and Eya2 have been independently implicated in the same types of cancer. Furthermore, coordinate overexpression of Six1and Eya2 significantly correlates with a dramatically shortened time to relapse and with shortened survival in breast cancers. These findings suggest that Eya and Six1 cooperate to stimulate breast tumorigenesis *and* progression. Because the Eya co-activator contains a unique protein phosphatase domain whose activity is required to activate Six1, it may serve as a novel anti-cancer drug target. However, an essential role for the Six1/Eya interaction and Eya's phosphatase activity in cancer cell proliferation and/or metastasis has not been formally proven. The above observations lead us to hypothesize that the Six1/Eya/DNA complex is an ideal drug target whose inactivation will inhibit tumor cell proliferation and metastasis in breast cancer. Because Six1 and Eya are embryonic genes with very limited expression in the adult, inhibitors of their expression/activity are likely to have limited side effects. To test this hypothesis, this proposal combines my (Dr. Ford's) strength in breast cancer biology with Dr. Rui Zhao's strength in structural biology/biochemistry. To determine whether Six1 activity can be targeted by modulating proteins within its transcriptional complex, we have begun to perform *in vitro* and *in vivo* assays to identify whether the co-factor of Six1, Eya2, and its phosphatase activity, is absolutely required for the ability of Six1 to induce tumorigenesis and metastasis. We are also attempting to solve the X-ray structure of the Six1/Eya/DNA ternary complex with the goal of setting the groundwork for designing structure-based inhibitors. Finally, to ensure that we will obtain inhibitors of the complex, we will perform high throughput screens (HTS) as a second method to identify small molecules that target the Six1-DNA or the Six1-Eya interaction, as well as Eya's phosphatase activity.

BODY

In the body of this progress report, we outline the progress made to date on each task of the original grant.

Task 1. Determine the role of Eya2 and its phosphatase activity in Six1-mediated breast tumorigenesis and metastasis (years 1 and 2). Work carried out in Dr. Ford's laboratory.

1A. Determine the effect of Eya2 knockdown on Six1-induced proliferation (months 1-6)

In this sub-aim, we wanted to determine whether loss of Eya2 in Six1 overexpressing MCF7 cells led to a decrease in proliferation. Previously, we had shown that proliferation was increased with Six1 overexpression [1] and that this increase was dependent on cyclin A1 activation by Six1. Preliminary experiments with Eya2 knockdown demonstrated that Six1 did depend on Eya2 for its ability to activate cyclin A1 (Fig.1). However, over time our Six1-overexpressing cells have changed such that they no longer proliferate more rapidly than the control cells in culture. We believe that this change may be due to the epithelial to mesenchymal transition (EMT) that the cells underwent in the presence of Six1-overexpression that occurred over time. Thus, we were unable to assess whether Eya2 knockdown reverses Six1-induced proliferation, as Six1-induced proliferation was lost in these cell lines. However, we were able to assess the role of Eya2 in Six1-induced EMT, a property that is associated with metastasis and will be outlined in Aim 1B below.

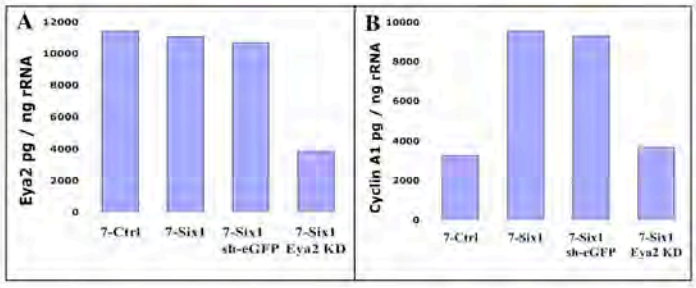


Fig. 1. Eya2 shRNA efficiently knocks down Eya2 in MCF7 Six1 cells, leading to a decrease in cyclin A1 levels of mRNAs determined by qRT-PCR.

1B. Determine the effect of Eya2 knockdown on transformation, tumor burden and metastasis (months 1-18)

We have made significant progress on this sub-aim, and our first manuscript that demonstrates that Eya2 is required for the ability of Six1 to mediate increased TGF-β signaling, EMT, increased stem cell capacity, and increased tumor initiating capacity is currently in press in *Oncogene*. I am attaching this manuscript to the current report. In this sub-aim, we stably knocked down Eya2 is MCF7-Six1 expressing cells and compared the Eya2 knockdown cells to MCF7-Six1 cells with a control shRNA and to MCF7-control (Ctrl) cells with a control shRNA. Fig. 1 of the manuscript shows the relative levels of Eya2 mRNA expression after real time RT-PCR in the cell lines containing shRNAs (2 different shRNAs were used, 2 clonal isolates were generally analyzed for each shRNA) vs control cell lines.

We have recently demonstrated that Six1 mediates metastasis via its ability to upregulate TGF-β signaling [2]. In addition, we have also shown that Six1 increases TGF-β signaling at least in part via upregulating the type 1 TGF-β receptor, TβRI (Micalizzi et al., *Cancer Res* 2010). We thus asked whether knockdown of Eya2 in Six1-overexpressing MCF7 cells could reverse the ability of Six1 to increase TβRI levels, and to activate TGF-β signaling. These data are shown in figure 2 of the manuscript, and demonstrate that Eya2 knockdown in Six1 overexpressing cells reverses the ability of Six1 to increase TβRI levels, to increase Smad3 levels, and to activate Smad-

mediated transcription, as assessed by the 3TP Smad-dependent reporter luciferase assay. Together, these data clearly demonstrate that Six1 requires Eya2 to activate TGF-β signaling.

Because we had previously demonstrated that Six1 induces an EMT in MCF7 cells that is dependent on its ability to activate TGF-β signaling, we went on to examine the role of Eya2 in Six1-induced EMT. Importantly, knockdown of Eya2 in Six1 expressing cells reverses the ability of Six1 to lead to an increase in the mesenchymal protein fibronectin, and reverses the ability of Six1 to re-localize E-cadherin and β-catenin, two adherens junction proteins, away from the membrane and into the cytosol (Fig 3 of the manuscript). Furthermore, Six1 is also dependent on Eya2 for its ability to lead to an increase in β-catenin mediated transcription, a hallmark of EMT (Fig. 3). Interestingly, however, Eya2 knockdown did not reverse the ability of Six1 to lead to decreased cell-matrix adhesion (Fig. 3D), a property that was also not reversed in Six1 overexpressing cells in which TGF-β signaling was downregulated (Micalizzi et al., *Cancer Res* 2010). These data strongly support a role for Eya2 in some, but not all properties of Six1-induced EMT, and suggest that Eya2 cooperates with Six1 to induce EMT phenotypes that are dependent on TGF-β signaling.

Because genes that induce EMT often also induce stem cell characteristics, and because we have obtained data that demonstrates that Six1 overexpression in MCF7 cells leads to increased cancer stem cells, as measured by flow cytometry, mammosphere assays, and *in vivo* tumor initiating transplant assays (Ritsuko and Ford, manuscript to be submitted this week), we asked whether Six1 was dependent on Eya2 to induce cancer stem cell characteristics. Indeed, knockdown of Eya2 in Six1-overexpressing cells reversed the increase in the breast cancer stem/progenitor pool as measured by flow cytometry for the $CD44^{+}/CD24^{lo}$ stem cell population (Fig. 4 of the attached manuscript). It also reversed the ability of Six1 to lead to increased mammosphere formation, an *in vitro* test for functional stem/progenitor cells (Fig. 4B and C).

We have also examined the dependence of Six1 on Eya2 in human breast cancers by both immunohistochemistry (IHC) and by examining microarray datasets. Our IHC analysis demonstrated that high Six1 and Eya2 levels correlate with high TGF-β signaling, as assessed by staining for nuclear Smad 3 (see figure 5 of the attached manuscript). By examining the Van de Vijver dataset of 295 breast cancer patients with early-stage invasive carcinoma [3], we have observed that while high Six1 in the absence of high Eya2 or high Eya2 in the absence of high Six1 do not predict shortened time to relapse, metastasis, or survival, having both high levels of Six1 AND Eya2 together do significantly correlate with shortened time to relapse and metastasis, and with shortened overall survival (Fig. 6). Indeed, these data are recapitulated in the Wang dataset of 286 node-negative breast cancers [4](supplemental figure 3). Together, these data strongly suggest that in human breast cancers, like in our model systems, Six1 is dependent on Eya2 to mediate its pro-tumorigenic and metastastic phenotypes. Furthermore, we have demonstrated that Eya1 has the same effect with Six1 in these datasets (figure 7 of the manuscript and supplemental figure 4), but that the other two Eya family members (Eya3 and 4) do not correlate to the same degree.

1C. Determine whether Eya's phosphatase activity is required for Eya's effect on Six1-mediated breast tumorigenesis/metastasis (months 12-24).

Although last year we were a bit ahead of schedule, this subaim has turned out to be more difficult then anticipated. We generated a number of cell lines with a rescue with an eya phosphatase dead mutant, but this mutant was not the active site mutant and therefore we needed to regenerate the lines. The lines are being remade, and we expect to test them now. Thus, this aim is a bit behind schedule but we expect to complete it in the near future.

Task 2. Identify small molecules that inhibit the Six1/Eya/DNA complex using structure-based and high throughput screen (HTS) approaches

This aim is primarily carried out by Dr. Zhao's laboratory with the one exception that any identified small molecules will be tested in cell culture by Dr. Ford's laboratory (they will first be tested biochemically by Dr. Zhao)

2A. Determine the crystal structure of Six1/Eya/DNA ternary complex for structure-based drug design

After generating, expressing, and purifying 22 Six1 constructs (4 truncation and 18 mutations) and two different Eya constructs, as well as trying many conventional crystal optimization techniques, we were not able to improve the initial Six1/ED/DNA crystals and large enough crystals to obtain usable diffraction data. However, we have made tremendous progress on determining the structure of the Six1/Eya2 Eya Domain (ED) complex. Using a MBP-Six1 fusion protein, we were able to crystallize MBP-Six1/ED that diffracted to 3.5Å at home (Fig. 2).

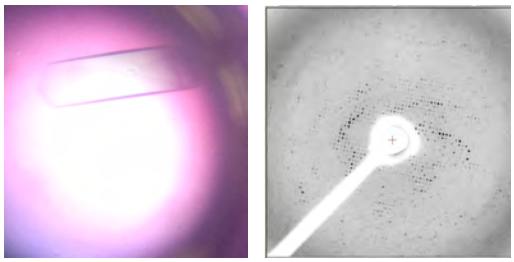


Fig. 2. Crystals (left) and diffraction pattern (right) of MBP-Six1/ED.

We collected a 2.5Å resolution data set at the synchrotron (Advanced Photon Source). Using the MBP and Eya2 ED as models, we determined the preliminary structure of MBP-Six1/ED (Fig. 3). In this preliminary structure, we can see electron density for MBP and ED very well. However, the electron density for Six1 is of poorer quality. We were able to build about 1/2 of the main chain and only about 1/6 of the side chains so far. To improve the electron density for Six1, we have produced Se-Met MBP-Six1/ED crystals and crystals of MBP-Six1/ED soaked with two heavy atom compounds (gold cyanide and Samarium acetate). We plan to collect Multi-wavelength Anomalous Dispersion (MAD) data set on these crystals soon at a synchrotron source. These data will enable us to determine the structure using the MAD method and improve the Six1 density, which will greatly facilitate model building. After model building is complete, we will carry out rounds of refinement to improve the model. Using the refined model, we will analyze the molecular details of the Six1/ED interaction. We will further verify the functional importance of these interaction details using mutagenesis and cell culture experiments. These details will be valuable for targeting the Six1/Eya interaction using structure-based drug design approach.

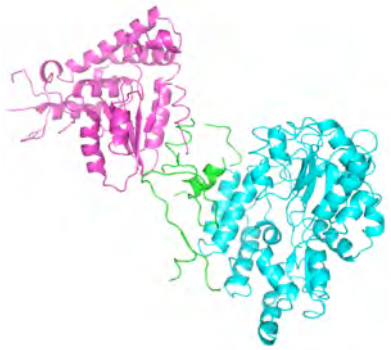


Fig. 3. Preliminary crystal structure of MBP-Six1/ED. MBP, Six1, and ED are in blue, green, and purple, respectively.

2B. **Identify small molecules that inhibit the Six1/Eya/DNA complex using HTS**

We have successfully developed a fluorescence-based Alpha-Screen assay to target the Six1/Eya protein/protein interaction (Fig. 4).

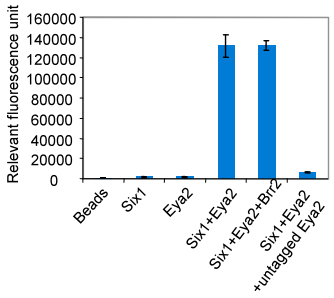


Fig. 4. Six1 and Eya2 produce a significant AlphaScreen signal only when together. Untagged Eya2 but not an irrelevant protein Brr2 competes with the Six1/Eya2 AlphaScreen signal.

In collaboration with Dr. Marc Ferrer at the NIH Chemical Genomics Center (NCGC), we carried out a pilot screen of the LOPAC library of 1,280 compounds (Sigma-Aldrich). The screen demonstrates that the assay has z' of 0.7 and signal/background of 8. These numbers indicate the robustness of the assay and its readiness for HTS. The screen identified six preliminary hits with IC50s ranging from 0.7-35mM (Fig. 5). We have developed an ELISA-based secondary assay to monitor the Six1/ED interaction and are in the process of confirming the preliminary hits using this secondary assay (Fig. 6a). We will also test whether these compounds inhibit a different protein-protein interaction (CtBP-E1A) as a test for the specificity of these compounds (Fig. 6b). Using the above preliminary data, we have applied and were just awarded a NIH R03 HTS grant to identify small molecule inhibitors of the Six1/Eya interaction using this assay we developed. With this grant, NCGC will screen over 300,000 compounds for us. We will carry out biochemical, structural, and cell culture experiments to characterize preliminary hits and optimize these hits in collaboration with NCGC.

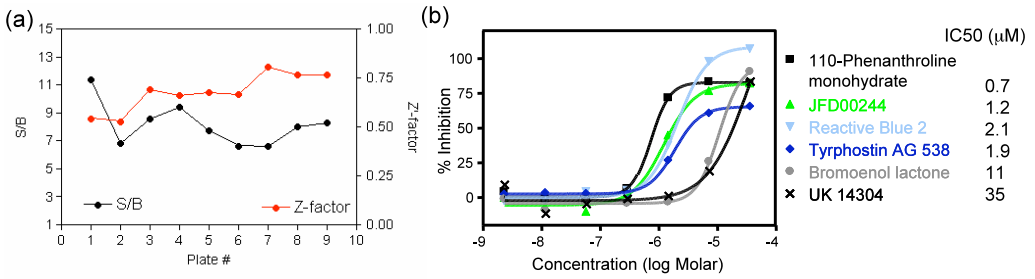


Fig. 5. Results of a test compound library screen in the AlphaScreen assay. (a) Statistics of the results from the LOPAC library screen in 9 plates in 1536-well format. (b) Six active compounds identified in the test screen.

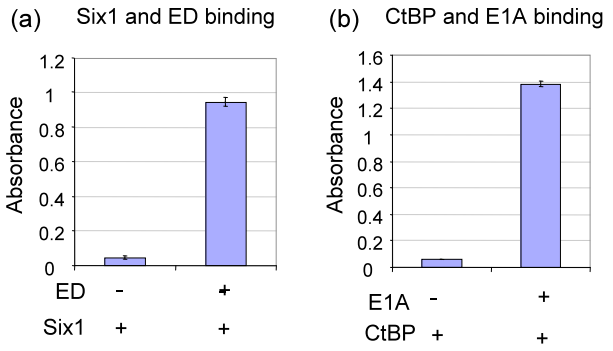
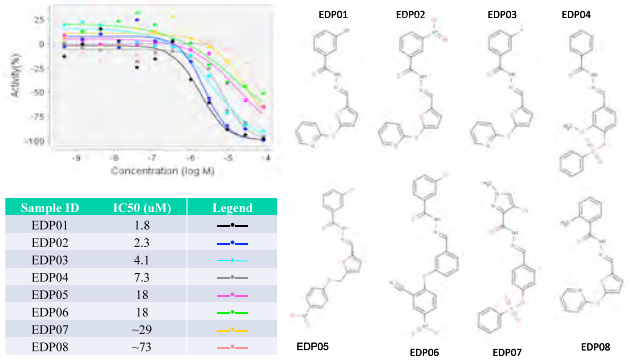


Fig. 6. ELISA as secondary and specificity assays. (a) Six1 and ED binding generates good signal in ELISA that can be used to validate hits primary screen. (b) CtBP and E1A binding demonstrated by ELISA can be used to examine the specificity of potential hits in disrupting Six1-ED interaction.

Last year, Pandy et al. demonstrated that Eya's phosphatase activity is important for breast cancer cell proliferation, transformation, migration, invasion, and metastasis [1]. We therefore decided to also develop HTS assays targeting the Eya phosphatase activity. We have successfully developed a fluorescence-based phosphatase assay using small molecule OMFP as substrate. We have applied and received a NIH R03 HTS grant which enabled us to screen over 300,000 compounds in collaboration with NCGC. The screen identified a class of eight structurally highly related compounds with IC50 from 1.5 to 75 mM (Fig. 7). We confirmed that these compounds indeed inhibit Eya's phosphatase activity

using a malachite-green based phosphatase assay (Fig. 8). We further demonstrated that these compounds do not inhibit other cellular phosphatases including PTP1B, PPM1A, and Scp1 (Fig. 8 and data not shown). We have reproduced crystals of ED which diffracted to 2.7Å resolution (Fig. 9). We are in the process of determining the structure of compound-soaked crystals as well as testing the effect of these compounds in cell culture experiments. NCGC has also synthesized 30 derivatives of the initial compounds. We plan to characterize these compounds as an effort to optimize these initial hits.



Sample ID	IC50 (uM)	Legend
EDP01	1.8	
EDP02	2.3	
EDP03	4.1	
EDP04	7.3	
EDP05	18	
EDP06	18	
EDP07	~29	
EDP08	~73	

Fig. 7. Large scale HTS identified a class of structurally highly related initial hits.

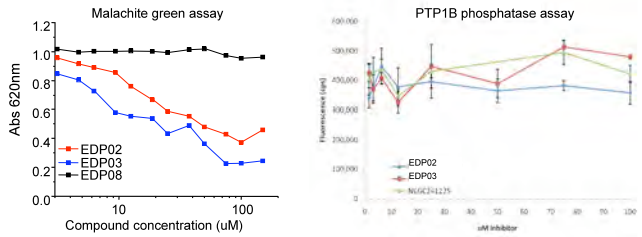


Fig. 8. A malachite green based secondary assay confirmed the top two initial hits do inhibit ED's phosphatase activity (left). These compounds do not inhibit the phosphatase activity of another cellular phosphatase PTP1B (right).

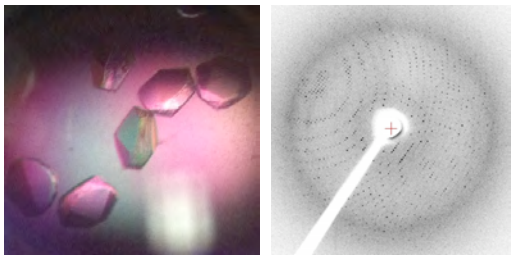


Fig. 9. Crystals (left) and diffraction pattern (right) of ED.

REPORTABLE OUTCOMES AIM1

Meeting Presentations (Susan Farabaugh, graduate student on project paid from her own DOD predoc award):

1. AACR Frontiers in Basic Research, October 2009
2. Annual Student Research Symposium, University of Colorado (one of the top prize winners for this presentation) 2009.
3. "Eya2 as Cofactor of Six1 in Human Breast Cancer" 24th Annual UC AMC Student Research Forum: Research Award for Outstanding Research (2010 poster presentation)
4. "Six1 is Dependent on its Coactivator Eya2 to Mediate Six1-Induced Breast Cancer Progression" UC-AMC & UC-Denver Research and Creative Activities Symposium Chancellor's Award of Excellence in Graduate Research (poster presentation 2010)
5. "Eya2 is Necessary for the Homeoprotein Six1 to Induce TGF-β Signaling, EMT Properties, and Stem Cell Characteristics" MRS-AACR Metastasis and Tumor Microenvironment conference, Philadelphia PA (2010).
6. "Eya2 is Necessary for Six1-Induce TGF-β Signaling, EMT Properties, and Stem Cell Characteristics". The NIH Graduate Student Research Festival, Bethesda MD (2010)

List of personnel receiving pay from this research effort:

Aaron Patrick, postdoctoral fellow
Erin Deitsch, technician
Barb Schiemann, past technician

Joshua Cabrera, technician

Patent:
Inhibitors of Eya2. UTEC.P0026US.P1, 1/10/11.

Funding resulting from Aim 1:
Breast Cancer Research Foundation-AACR (Ford PI, Zhao Co-I) 10/01/10 - 9/30/12
Targeting the Six1/Eya Complex for Anti-Breast Cancer Metastasis Therapies $90,500/yr

Major Goals: The major goal of this proposal is test small molecules against the Eya phosphatase activity for their ability to inhibit Six1/Eya mediated transcription and tumorigenicity/metastasis and to begin to develop small molecule inhibitors against the Six1/Eya interaction.

Manuscripts:

Farabaugh, S.M., Micalizzi, D.S., Jedlicka, P., **Zhao, R**., and **Ford, H.L.** (2011). Eya2 Is Required to Mediate the Pro-Metastatic Functions of Six1 Via the Induction of TGF-β Signaling, Epithelial-Mesenchymal Transition, and Cancer Stem Cell Properties. In press, *Oncogene*.

Degrees obtained:

1. Aaron Patrick obtained PhD in November 2009 from the Molecular Biology Program at The University of Colorado School of Medicine in part on his crystallography work performed for this proposal.
2. Susan Farabaugh obtained PhD in December 2010 from the Biochemistry Program at The University of Colorado School of Medicine on her work performed for this proposal.

REPORTABLE OUTCOMES AIM2

Meeting Presentations:
Krueger, A., Patrick, A., Ford, H., Zhao, R. Identify small molecule inhibitors of the Six1 transcriptional complex for anti-breast cancer drug design. American Association for Cancer Research 100th Annual Meeting, Denver, Colorado, April 2009.

Colorado Science Conference, Denver, Colorado, November 2009. Targeting the Six1/Eya transcriptional complex for anti-breast cancer drug design.

Krueger, A., S. Dehdashti, W. Zheng, S. Patnaik, J. Marugan, N. Southall, H. Ford, R. Zhao. Identification and characterization of small molecule inhibitors targeting Eya phosphatase activity for anti-breast cancer therapy. American Association for Cancer Research annual meeting, Orlando, Florida, April 2011.

Blevins, M., M. Swaroop, M. Ferrer, J. An, S. Jones, H. Ford, and R. Zhao. Identification of Potential Anti-Tumor Therapeutics Targeting the Six1 Transcriptional Complex. American Association for Cancer Research annual meeting, Orlando, Florida, April 2011.

List of personnel receiving pay from this research effort:
Aaron Patrick, postdoctoral fellow
Aaron Krueger, graduate student
James Shanks, technician
Melanie Blevins, graduate student

Patent:
Inhibitors of Eya2. UTEC.P0026US.P1, 1/10/11.

Funding:
National Institute of Health R03 MH 90869-01 (PI: Rui Zhao and Heide Ford)
6/1/10-5/31/12 $25,000/year direct costs
Title: Identify inhibitors of the Eya phosphatase activity using high throughput screening

National Institute of Health R03 MH 95583-01 (PI: Zhao and Ford)
awarded but not yet funded
Title: Identify inhibitors of the Six1/Eya interaction using high throughput screening

Data deposited:
Eya phosphatase inhibitor screening results have been deposited to pubchem.

Degrees obtained
Aaron Patrick obtained PhD in November 2009 from the Molecular Biology Program at The University of Colorado School of Medicine in part on his crystallography work performed for this proposal.

CONCLUSION

To date, our data clearly demonstrate that Eya2 is required for the ability of Six1 to induce both tumorigenic and metastatic properties. These data are important because Eya2 may make a very good anti-breast cancer drug target. Because Six1 is a transcription factor, it will likely not be targetable with small molecule inhibitors. However, since Eya2 is required for Six1-mediated effects, we may be able to target the Six1/Eya interface as an anti-breast cancer therapy. Since the phosphatase activity of Eya is now implicated in breast cancer metastasis, the phosphatase activity will also be a drug target for small molecule inhibitors. In fact, as outlined in the progress report for Aim2, our data demonstrate that we can determine the Six1/Eya crystal structure which will be a valuable resource for structure-based drug design targeting the Six1/Eya interface as well as for optimizing initial hits identified from high throughput screening. We have developed an AlphaScreen assay to monitor the Six1/Eya interaction and were awarded a NIH R03 HTS grant to screen over 300,000 compounds in the near future. We have developed HTS assays targeting the Eya phosphatase activity, applied and received a NIH

R03 HTS grant, screened over 300,000 compounds, obtained a class of highly promising initial hits, and are in the process of characterizing and optimizing these hit. Within the next year, we plan to complete structural determination of Six1/ED, carry out large-scale HTS of Six1/ED interaction, and complete characterization of the Eya phosphatase inhibitors. This work should generate 3-4 more publications within the next year. We are well on our way to obtain effective inhibitors of the Six1/Eya complex by targeting the Six1/ED interaction and the Eya phosphatase activity. Targeting the Six1/Eya complex is novel because this complex is critical for normal embryonic development, but is not believed to be required by adult, differentiated cells (in fact, the two proteins are not expressed in many adult tissues). Thus, targeting this transcriptional complex has the ability to inhibit the tumor on multiple fronts while conferring limited side effects. Such breast cancer targets are badly needed and it is for this reason that we are interested in carrying out the work described in this proposal.

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2 Micalizzi, D. S. *et al.* The Six1 homeoprotein induces human mammary carcinoma cells to undergo epithelial-mesenchymal transition and metastasis in mice through increasing TGF-beta signaling. *J Clin Invest* **119**, 2678-2690, doi:37815 [pii] 10.1172/JCI37815 (2009).

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4 van de Vijver, M. J. *et al.* A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* **347**, 1999-2009 (2002).

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Eya2 Is Required to Mediate the Pro-Metastatic Functions of Six1 Via the Induction of TGF-β Signaling, Epithelial-Mesenchymal Transition, and Cancer Stem Cell Properties

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Abstract

Six1 is a critical regulator of embryonic development that requires interaction with the Eya family of proteins (Eya1-4) to activate the transcription of genes involved in neurogenesis, myogenesis, and nephrogenesis. While expression of Six1 and Eya family members is predominantly observed in development, their overexpression is observed in numerous cancers. Importantly, both Six1 and Eya have independently been shown to mediate breast cancer metastasis, but whether they functionally interact during tumor progression has not been explored. Herein we demonstrate that knockdown of Eya2 in MCF7 mammary carcinoma cells reverses the ability of Six1 to induce TGF-β signaling, as well as to induce characteristics associated with epithelial-mesenchymal transition (EMT) and cancer stem cells (CSCs), suggesting that Six1 is dependent on Eya2 to mediate numerous pro-metastatic characteristics. The importance of the Six1/Eya interaction in human breast cancer is underscored by the finding that high levels of Six1 correlate with shortened time to relapse and metastasis as well as decreased survival only when co-expressed with high levels of Eya2. Overall, these data implicate Eya2 as a necessary cofactor for many of the metastasis promoting functions of Six1, suggesting that targeting the Six1/Eya interaction may inhibit breast cancer progression. Since Six1 and Eya2 are not highly expressed in most adult tissues, the Six1-Eya interaction may be a valuable future therapeutic target whose inhibition would be expected to impair breast cancer progression while conferring limited side effects.

Keywords: Six1, Eya, TGF-β, epithelial-mesenchymal transition, cancer stem cells

Introduction

Many processes necessary for early embryonic development are recapitulated in cancer, often as a result of homeobox gene re-expression (Cillo *et al.*, 1999; Ford, 1998; Samuel and Naora, 2005). Such inappropriate expression of homeobox genes, which encode transcription factors, allows for the acquisition of early developmental phenotypes, such as proliferation, survival, invasion and migration in adult cells, thereby contributing to tumorigenesis and/or tumor progression (Abate-Shen, 2002). The Six1 homeobox gene plays a critical role in the development of a number of organs via promoting proliferation, survival, migration, and invasion of precursor cell populations (Laclef *et al.*, 2003a; Laclef *et al.*, 2003b; Xu *et al.*, 2003; Li *et al*., 2003). In muscle development, Six1 contributes to the delamination and migration of myogenic precursor cells from the dermomyotome to the developing limb, in a process that entails an epithelial-mesenchymal transition (EMT)(Laclef *et al.*, 2003a; Laclef *et al.*, 2003b; Xu *et al.*, 2003). In addition to the role of Six1 in development, recent work has identified misexpression of Six1 in numerous cancers including breast (Ford *et al.*, 1998; Reichenberger *et al.*, 2005), ovarian (Behbakht *et al.*, 2007), cervical (Micalizzi *et al.*, 2009)**,** hepatocellular carcinoma (Ng *et al.*, 2006), as well as Wilms' tumors (Li *et al.*, 2002) and alveolar rhabdomyosarcomas(Yu *et al.*, 2004). Importantly, Six1 overexpression in cancer recapitulates the pro-proliferative, pro-survival, and pro-migratory phenotypes attributed to Six1 in development (Behbakht *et al.*, 2007; Coletta *et al.*, 2004; Micalizzi *et al.*, 2009; Yu *et al.*, 2004).

Six1 belongs to the evolutionarily conserved Six1-Eya-Dach transcriptional regulatory network that is critical during embryonic development. Members of the Eya family of Six1 coactivators (Eya1-4) play critical roles in Six1-mediated transcriptional activation. Six1 and Eya1 knockout

mice phenocopy each other, and both molecules are necessary for the proper development of ears, muscle, kidney, thymus, and sensory neurons, as well as for overall neonatal survival (Ando *et al.*, 2005; Laclef *et al.*, 2003b; Li *et al.*, 2003; Xu *et al.*, 2003; Xu *et al.*, 2002). The phenotypes observed in both KO mice are, at least in-part, due to poor progenitor cell proliferation and survival, underscoring the role of the Six1-Eya complex in the expansion of progenitor cell populations during normal development. The role of the Six1-Eya complex in human disease is also clear, as both Six1 and Eya1 mutations can be found in branchio-oto-renal syndrome, where mutations disrupt individual protein function, Six1-DNA binding, and Six1-Eya1 binding (Abdelhak *et al.*, 1997a; Abdelhak *et al.*, 1997b; Kochhar *et al.*, 2008; Orten *et al.*, 2008; Patrick *et al.*, 2009; Ruf *et al.*, 2004; Vincent *et al.*, 1997). Additionally, Eya4 mutations are observed in sensorineural hearing loss (SNHL) (Schonberger *et al.*, 2005; Wayne *et al.*, 2001; Zhang *et al.*, 2004) where Eya4 function and Six1-Eya4 interactions are lost. Together, data from both mouse models and human disease underscore the critical developmental function of the Six1-Eya interaction.

Although not highly expressed in most adult tissues, Six1 is misexpressed in multiple cancers and re-instates a pro-proliferative and pro-survival phenotype(Coletta *et al.*, 2004; Yu *et al.*, 2006). In breast cancer, Six1 also induces EMT- and stem cell-like phenotypes, the former of which is mediated through upregulating TGF-β signaling (Micalizzi *et al.*, 2009) and is likely critical for Six1-induced metastasis. Six1 correlates with advanced disease in many types of cancer, including breast (Micalizzi *et al.*, 2009), ovarian (Behbakht *et al.*, 2007), and alveolar rhabdomyosarcomas (Yu *et al.*, 2004). Overexpression of Eya1 and Eya2 family members have been identified in many of the same cancers as Six1(Li *et al.*, 2002; Zhang *et al.*, 2005). In

ovarian cancer, two independent studies have demonstrated that Six1 and Eya2 each correlate with poor patient survival in advanced disease (Behbakht *et al.*, 2007; Zhang *et al.*, 2005); however, as of yet, the role of Six1 and Eya *together* in cancer has not been studied. Importantly, Eya was recently shown to increase proliferation, migration, invasion, transformation, and metastasis in mammary carcinoma cells (Pandey *et al.*, 2010); suggesting that Eyas, like Six1, play an important role in breast cancer and that they may in fact cooperate with Six1 to confer pro-proliferative and migratory phenotypes. While a non-nuclear function of Eya was postulated to mediate its pro-tumorigenic phenotypes (Pandey *et al.*, 2010), no formal experiments were conducted to test the relevance of the interaction between Six1 and Eya in breast tumorigenesis and/or metastasis.

In this study, we demonstrate a cooperative role between Six1 and Eya in mediating phenotypes associated with breast tumorigenesis and metastasis. Knockdown of Eya2 in Six1 overexpressing MCF7 mammary carcinoma cells reverses Six1-mediated induction of TGF-β signaling, as well as the ability of Six1 to induce EMT and an increase in cancer stem cell characteristics. The relevance of the Six1-Eya interaction in human breast cancer is underscored in gene expression datasets, where only co-expression of Six1 and Eya can predict poor patient prognosis. Taken together, these data strongly support the hypothesis that Eya is a critical activator of Six1 in human breast cancer, where it is required to mediate Six1-induced TGF-β-dependent EMT and expansion of the cancer stem-like cell population.

Results

Eya2 is the predominant Eya in MCF7 cells

We have previously shown that Six1 overexpression in the MCF7 mammary carcinoma cell line activates the TGF-β pathway (Micalizzi *et al.*, 2009), leading to a loss of epithelial and gain of mesenchymal characteristics. To determine whether Six1 requires the Eya family of cofactors to mediate these phenotypes, we first examined which Eyas, if any, are expressed in MCF7 cells. Using qRT-PCR with plasmid standard curves and primers that specifically recognize individual Eya members, we determined the mRNA copy number of each Eya in MCF7 cells overexpressing Six1 (MCF7-Six1) and control (MCF7-Ctrl). Importantly, we identified Eya2 as the predominant Eya family member in MCF7 cells, exhibiting 5-fold greater expression than Eya3. In contrast, Eyas 1 and 4 were absent or expressed only at very low levels (Fig1a). To determine the dependence of Six1 phenotypes on Eya2 function, we stably and specifically knocked down Eya2 in MCF7-Six1 cells using two independent shRNA sequences. Independent control cell lines were also established in which a scramble shRNA control was introduced into MCF7-Six1 and MCF7-Ctrl cells. Two clonal isolates were propagated from each shRNA in each line to control for insertion site effects. Eya2 mRNA and protein levels were determined in each clone (Fig1b and c), and on average, we achieved a 75% knockdown.

Eya2 knockdown in MCF7-Six1 cells reverses the ability of Six1 to induce TGF-β signaling

We have previously shown that Six1 activates the TGF-β signaling pathway when overexpressed in MCF7 cells (Micalizzi *et al.*, 2009; Micalizzi *et al.*, 2010). Using our Eya2 knockdown cells, we examined the dependence of Six1 on Eya2 in mediating increased TGF-β signaling. Because we have observed that Six1 overexpression leads to an increase both in the levels of the Type I

TGF-β receptor (TβRI) (Micalizzi *et al.*, 2010) and in total Smad3 levels (Micalizzi *et al.*, 2009), each which may contribute to the overall increase in TGF-β signaling, we first examined whether loss of Eya2 would reverse the Six1-mediated increases in these protein levels. Indeed, Eya2 knockdown reverses the Six1-mediated increase in the levels of TβRI and Smad3 (Fig2b). Further, Eya2 knockdown in MCF7 cells leads to decreased signaling downstream of TGF-β as measured by 3TP-luciferase activity, a measurement of Smad-mediated TGF-β responsive transcription (Fig2c). Finally, co-expression of Six1 and Eya2 increases both total and p-Smad3 levels in NMuMG cells above that observed with expression of Six1 or Eya2 alone, suggesting that the two proteins cooperate to activate TGF-β signaling in multiple contexts (Supplemental Fig1). Together, these data demonstrate that Eya2 is necessary for the ability of Six1 to activate TGF-β signaling.

Eya2 knockdown partially reverses the ability of Six1to induce EMT

In both cell culture and animal models, Six1 overexpression results in the loss of the epithelial phenotype with a concomitant gain in the mesenchymal phenotype (McCoy *et al.*, 2009; Micalizzi *et al.*, 2009). We have previously shown that this EMT requires Six1-induced TGF-β signaling (Micalizzi *et al.*, 2009), and thus asked whether Six1 is also dependent on Eya2 to mediate EMT. In support of the hypothesis that Six1 requires Eya2 to mediate an EMT, knockdown of Eya2 in MCF7-Six1 cells reverses the Six1-induced increase in the mesenchymal marker fibronectin (Fig3a). Additionally, Eya2 is required for the Six1-induced re-localization of E-cadherin and β-catenin away from the insoluble (membranous) fraction and into the soluble (cytoplasmic/nuclear) fraction, a hallmark of EMT (Fig3b and 3c). As expected, the decreased levels of soluble β-catenin in the presence of Eya2 knockdown correlates with decreased Six1-

induced β-catenin-responsive transcription as measured by TOP-FLASH reporter activity (Fig3d). These data demonstrate that Eya2 is required for the ability of Six1 to induce and/or maintain features of EMT associated with the induction of mesenchymal properties. However, loss of Eya2 did not restore cytokeratin 18 expression to MCF7 cells, which is normally downregulated in the presence of Six1 (data not shown), nor did it reverse the decrease in cell-matrix adhesion observed with Six1 overexpression (Fig3e). This suggests that either: 1) Eya2 is not globally required for Six1-induced EMT, or 2) that some Six1-induced characteristics of EMT are permanent once induced and no longer dependent on Six1 function.

Six1 is dependent on Eya2 for enhancement of cancer stem/progenitor cell characteristics

We recently demonstrated that Six1 promotes a stem/progenitor cell phenotype when overexpressed in mouse mammary glands and that tumors which arise in these transgenic mice appear to be derived from a stem/progenitor cell population (McCoy *et al.*, 2009; Micalizzi *et al.*, 2009). Additionally, Six1 drives TGF-β signaling and EMT, properties that are associated with cancer stem-like cells (CSCs) (McCoy *et al.*, 2009; Micalizzi *et al.*, 2009; Ouyang *et al.*; Taylor *et al.*). Thus, we set out to test whether Eya2, which is required for the ability of Six1 to induce TGF-β signaling and EMT, is also required for the ability of Six1 to induce a cancer stem cell-like phenotype. While Six1 overexpression of MCF7 cells leads to an *increase in* the population of cells that are CD44+ and CD24-, markers of mammary stem/progenitor cells (Al-Hajj *et al.*, 2003), loss of Eya2 significantly reverses this increase (Fig4a and Supplemental Fig2). Furthermore, knockdown of Eya2 reverses the increase in functional cancer stem-like cells observed with Six1 overexpression, as demonstrated in secondary tumorsphere formation assays (Fig4b). Interestingly, Six1 overexpression not only increases the number of tumorspheres

formed by MCF7 cells, but it significantly alters their morphology from a uniformed sphere to a less organized shape, a phenotype that is also reversed with Eya2 knockdown (Fig4c). Together, these results demonstrate that Eya2 is necessary for the ability of Six1 to induce characteristics of cancer stem/progenitor-like cells.

Co-expression of Eya2 and Six1 correlates with activated TGF-β signaling in human breast cancer

We previously demonstrated that Six1 induces metastasis via increasing TGF-β signaling. Additionally, we have shown that high levels of Six1 correlate with high levels of activated TGF-β signaling as well as with adverse outcomes in human breast cancer (Micalizzi *et al.*, 2009). Based on our results suggesting that Eya2 is required for Six1-activated TGF-β signaling, we performed immunohistochemical analysis on breast cancer tissue arrays to determine if Six1 is dependent on Eya2 to mediate TGF-β signaling in human breast cancer. We first produced and characterized an Eya2-specific antibody using an N-terminal Eya2 peptide. Importantly, this newly developed antibody specifically recognizes Eya2 as opposed to other Eya family members (Supplemental Fig3). Using tissue arrays containing fifty malignant primary breast tissue samples, immunohistochemical analysis was performed with antibodies against Eya2, Six1, and Smad3, which when nuclear is an indicator of active TGF-β signaling. Following blinded scoring, the data were divided into above the mean and below the mean nuclear staining for Six1 and Eya2. Co-expression of high levels of Six1 and Eya2 significantly correlates with nuclear localized Smad3 (Fig5), supporting a critical cooperative role for Six1 and Eya in mediating TGF-β signaling in human breast cancer.

Co-expression of Six1 and Eya2 correlates with advanced disease and poor prognosis in human breast cancer

Because Six1 requires Eya2 to mediate increased TGF-β signaling and to promote EMT and cancer stem cell-like properties, and because Six1 and Eya2 correlate with activated TGF-β signaling in human breast cancer, which we have previously shown is essential for Six1-induced metastasis, we further examined whether Six1 is dependent on Eya2 to predict poor prognosis in human breast cancer. Using the van de Vijver gene expression dataset, profiling tumor mRNA from 295 women with early-stage invasive breast carcinoma (van 't Veer *et al.*, 2002), we previously demonstrated that high Six1 levels significantly correlate with poor patient prognosis as measured by shortened time to relapse, shortened time to metastasis, and decreased breast cancer specific survival (McCoy *et al.*, 2009; Micalizzi *et al.*, 2009). However, in this previous analysis, the correlation with Eya2 was not investigated. We now show that high levls of Eya2 also significantly correlate with shortened time to relapse, time to metastasis, and decreased survival (Supplemental Fig4). However, in breast cancer patients whose tumors expressed high Six1 levels in the absence of high Eya2, as well as high Eya2 levels in the absence of high Six1, there is NO correlation with any of these parameters. In contrast, only when tumors express BOTH high levels of Six1 and Eya2 together, is a strong significant correlation found with shortened time to relapse, shortened time to metastasis, and with shortened survival (Fig6). These results can be corroborated in the independent Wang dataset (Wang *et al.*, 2005), consisting of 286 lymph-node negative breast cancer patients (Supplemental Fig5). These data strongly suggest that Six1 and Eya2 cooperate in human breast cancer and reinforce the hypothesis that Eya2 is required for the ability of Six1 to mediate tumor progression.

As Eya1, Eya2, and Eya4 have all been implicated in cancer, we extended our analysis of the van de Vijver dataset to examine whether any Eya family member is able to cooperate with Six1 in human breast cancer, or whether this cooperation is specific to Eya2. Importantly, in the van de Vijver dataset, Eya1 in conjunction with Six1 also significantly associates with shortened time to relapse and to metastasis, as well as with shortened survival (Fig7), and although not significant, Eya1 does show a cooperative trend with Six1 in the Wang dataset (Supplemental Fig6). In comparison, Eya3 only correlates with survival in the van de Vijver dataset and Eya4 does not correlate with any adverse outcome in conjunction with Six1 in either dataset (data not shown). Together, these data strongly suggest that Eya1 and Eya2 are both able to cooperate with Six1 in human breast cancer.

Discussion

The Six homeobox transcription factor reactivates developmental pathways out of context in numerous tumor types, likely contributing to both tumor initiation and tumor progression. Downstream pathways involved in, and required for, Six1-activated tumor initiation and tumor progression have been studied intensely over the past few years (Coletta *et al.*, 2004; Micalizzi *et al.*, 2009; Micalizzi *et al.*, 2010; Yu *et al.*, 2006). Since Six1 has no intrinsic activation or repression domains, we hypothesized that it would require a co-factor(s) to mediate its tumor promotional characteristics. Because members of the developmentally required Eya coactivator family are overexpressed in many of the same cancers as Six1, including ovarian cancer where both Six1 and Eya2 correlate with advanced-disease and poor patient survival (Behbakht *et al.*, 2007; Zhang *et al.*, 2005), we pursued Eya proteins as relevant required co-factors of Six1 in human breast cancer.

To establish if Six1 requires Eya to mediate its tumorigenic effects, we knocked down Eya2 in MCF7 mammary carcinoma cells overexpressing Six1. In this study, we demonstrate for the first time, a cooperative role for Six1 and Eya in mediating the pro-metastatic phenotypes induced by Six1. Eya2 is required by Six1 to activate TGF-β signaling, as well as to induce both EMT and cancer stem cell-like characteristics. These data are supported clinically by the demonstration that Six1 and Eya2 are co-expressed with activated TGF-β signaling, and that the two proteins together, but not individually, correlate with shortened time to relapse and metastasis and with decreased survival. These results strongly suggest that Eya2 and Six1 act in concert to promote breast tumor progression**.**

Although all Eya family members cooperate with Six1 to mediate Six1-induced transcription in cell culture models (Li *et al.*, 2003; Ohto *et al.*, 1999; Zhang *et al.*, 2004), we demonstrate that only Eya1 and Eya2 strongly collaborate with Six1 in human breast cancer. This association of Six1 with Eya1 and Eya2 is not surprising as Eya1 and Six1 knockout mice phenocopy each other (Ando *et al.*, 2005; Laclef *et al.*, 2003b; Li *et al.*, 2003; Xu *et al.*, 1999; Xu *et al.*, 2003) and as Eya2 and Six1 both correlate with advanced disease and decreased survival in ovarian cancer (Behbakht *et al.*, 2007; Zhang *et al.*, 2005). In contrast, overexpression of Eya3 has not been observed in cancer, nor has an endogenous role for Eya3 with Six1 been demonstrated. Interestingly, Eya4 is methylated and downregulated in gastrointestinal cancers (Osborn *et al.*, 2006; Zou *et al.*, 2005), but is overexpressed in malignant peripheral nerve sheath tumors along with other Eya and Six family members (Miller *et al.*, 2010), suggesting that Eya4 may play roles in both tumor suppression and progression, depending on the context. Thus, while Eya1 and Eya2 appear to be the key Eya family members necessary for Six1-mediated tumor

progression in the breast, we hypothesize that, given the correct context, any Eya family member may be sufficient to cooperate with Six1 to mediate tumorigenesis.

Interestingly, a recent study demonstrates that overexpression of Eya in MCF7 mammary carcinoma cells promotes proliferation, transformation, migration, and invasion of breast cancer cells, while knockdown of Eya3 in MDA-231 metastatic mammary carcinoma cells inhibits invasion, migration, and metastasis in nude mice (Pandey et al., 2010). The authors of this recent study suggest that Eya promotes tumorigenic phenotypes largely through cytoplasmic functions, since nuclear targeted Eya did not induce transformation, migration or invasion of breast cancer cells to the same extent as wild type Eya protein. While a cytoplasmic function for Eya likely contributes to its ability to mediate tumorigenic and metastatic phenotypes, the cell lines used in this study contain low levels of Six1, making it difficult to assess the dependence of Eya on Six1 in this context. It has previously been shown that the presence of Six family members significantly affects the percentage of Eya proteins located in the nucleus versus the cytoplasm (Ohto *et al.*, 1999), and that the cytoplasmic/nuclear Eya ratio is further influenced by expression of Abl and G-alpha$_i$ proteins, which function with Eya in the cytoplasm (Embry *et al.*, 2004; Fan *et al.*, 2000; Xiong *et al.*, 2009). Interestingly, complete eye restoration in *Drosophila* requires balanced nuclear and cytoplasmic *eya* expression (Xiong et al., 2009), suggesting that full Eya function may require spatial regulation between the two Eya pools. Indeed, while the cytoplasmic function of non-targeted Eya was deemed critical for the pro-tumorigenic and metastatic properties observed in the aforementioned study, nuclear targeted Eya still increased pro-tumorigenic/metastatic phenotypes 3-fold above baseline (Pandey *et al.*, 2010), suggesting that the cytoplasmic and nuclear functions of Eya may cooperate to impart the tumorigenic

response. Importantly, the requirement of a direct Six1-Eya interaction in mediating metastatic phenotypes, as opposed to the two proteins working in parallel cooperative pathways, is not yet known. Nonetheless, the cell culture models used in this study are strongly supported by the clinical data published herein, and indicate that Six1 and Eya indeed require each other to mediate breast cancer progression.

In addition to its transactivation activity, Eya contains two phosphatase activities, N-terminal serine/threonine phosphatase activity and C-terminal tyrosine phosphatase activity. The C-terminal tyrosine phosphatase activity acts in the nucleus to activate a repair and survival pathway by dephosphorylating H2AX in response to DNA damage (Cook *et al.*, 2009; Krishnan *et al.*, 2009) and in the cytoplasm to mediate phosphotyrosine signaling networks during *Drosophila* development by interacting with the Abelson tyrosine kinase (*abl*)(Xiong *et al.*, 2009). The N-terminal serine/threonine phosphatase activity plays a role in the cytoplasm, where it is required for the ability of Eya4 to enhance the innate immune response (Okabe *et al.*, 2009). Significantly, the tyrosine phosphatase activity of Eya is necessary for Eya-induced migration, invasion, transformation, and metastasis (Pandey *et al.*, 2010). The requirement for Eya phosphatase activity in mediating transcriptional activation, specifically through Six1, has not been completely elucidated, particularly in mammalian cells. Early in-vitro studies analyzing activation of Six1 reporter promoters suggest that the Eya phosphatase activity is important to co-activate transcription through Six1 (Li *et al.*, 2003). However, a more recent study in *Drosophila* suggests Eya phosphatase activity is only required for a subset of Six1 transcriptional targets (Jemc and Rebay, 2007). Thus, the role of Eya phosphatase activity in mediating Six1-induced pro-metastatic phenotypes remains to be determined.

In conclusion, we have found that the Eya co-activator is required for the ability of Six1 to mediate a number of pro-metastatic properties, and that the two molecules together significantly predict adverse outcomes in human breast cancer. Understanding the necessity of Six1 on both a direct interaction with Eya and on the Eya phosphatase activity is important for future development of anti-cancer agents that target the Six1-Eya complex. Six1 and Eya2 are implicated in advanced breast cancer and are not normally expressed in most adult tissues. Thus, inhibiting these proteins in breast, ovarian, and other carcinomas may result in a therapeutic agent that would target tumor progression with limited side effects to patients.

Methods

Cell culture

One MCF7-Six1 and one MCF7-Ctrl stable clone (Ford *et al.*, 1998) was stably transfected using Effectene (Qiagen, Valencia, CA, USA) with 5 shRNA constructs and one scramble negative control in the SureSilencing pGeneClip vector (SABiosciences, Frederick, MD, USA). Cells were selected with 2.5ug/ml puromycin and 2 individual clonal isolates chosen from each of two working Eya2 shRNA constructs (shRNA1: CGTGCGCATTGGCCTTATGAT; shRNA2: GGGTTCTATCAAGGAGGAAAT), as well as 2 scrambled control clones (GGAATCTCATTCGATGCATAC).

Real Time PCR

Total RNA was TRIzol isolated and RNeasy mini kit purified (Qiagen). Quantification to compare Eya mRNA levels was performed using plasmid standard curves and calculation of copy number for each Eya mRNA. Relative expression was used for comparison of Eya2 levels between shRNA clones and determined by ΔΔCT method (Livak and Schmittgen, 2001). Supplemental Figure7 lists primer and probes.

Antibody Production

An Eya2 antibody was produced by Proteintech Group, Inc (Chicago, IL). An N-terminal Eya2 peptide (aa17-37: LDKLKFNRADAAVWTLSDRQG) was KLH conjugated. 1mg of peptide was injected on day1 with boosts on days 28, 40, 58, and 76 and bleeds on days 72 and 102. The antibody was antigen affinity purified and tested on lysates from MCF7 cells transfected with Eya2 or Eyas 1,3,4 for control.

Immunofluorescence

Cells were grown on glass slides, fixed with formaldehyde, permeablized with 0.5% Triton X-100/PBS, incubated with Eya2 antibody (1:100; Ford Lab), then with Texas Red-conjugated antirabbit IgG antibody (Sigma-Aldrich, St. Louis, MO) and stained with DAPI.

Western blot analysis

Western blot analysis was performed on whole cell lysates made with RIPA buffer(Ford *et al.*, 2000). Primary antibodies used were: E-cadherin, β-catenin, and Fibronectin (BD Biosciences, San Diego, CA, USA), β-actin (Sigma-Aldrich), Smad3 (Invitrogen, Carlsbad, CA, USA) Eyas 1, 3, 4 (SantaCruz Biotechnology, Santa Cruz, USA). Cell fractionation performed as previously described (Shtutman *et al.*, 2006).

3TP and TOP-flash luciferase

Eya2 and scramble shRNA clones were plated in triplicate at 110,000 cells/well in a 12-well plate. After 24 hours, cells were cotransfected with 3TP (Wrana *et al.*, 1992) or pTOP-flash plasmid (Korinek *et al.*, 1997), and a renilla luciferase construct containing a cryptic promoter, using FuGENE 6 (Roche, Indianapolis, IN, USA). After 48 hours, lysates were prepared and analyzed with the Dual Luciferase Kit (Promega, Madison, WI, USA) on a Modulus Microplate (Turner Biosystems, Sunnyvale, CA, USA).

Cell adhesion

For cell adhesion, 96-well plates coated with laminin, fibronectin, collagen I, or collagen IV (BD Biosciences, Biocoat) were blocked with 1%BSA for 1 hour. 2.5×10^4 cells were added,

incubated 1 hour at 37º, and then washed 3 times with PBS, fixed for 10 minutes with cold methanol, stained with 0.04% crystal violet, and solubilized with 10% glacial acetic acid. Absorbance was determined at 570 nm on a microplate reader (Molecular Devices, Sunnyvalle, CA, USA).

Cancer Stem Cell Assays

$7.5x10^5$ cells were plated and grown for 48 hours. For flow cytometry analysis, cells were trypsinized, washed with 0.5%BSA-PBS, stained with anti-CD44-APC and anti-CD24-biotin (BD Biosciences) (1:100) for 30 minutes, then stained with anti-streptavidin-V450 (BD Biosciences) (1:1000) for 30 minutes, and resuspended in 400 µl of 0.06 µg/ul PI/0.5% BSA-PBS. Fluorescence was detected with CyAn (Beckman Coulter). For tumorsphere assays, cells were trypsinized and re-plated at 1,000 cells/ml in 6-well, ultra-low attachment plates (Fisher-Scientific) in 2 mls of serum-free DMEM/F12 media (Hyclone), supplemented with 20ng/ml bFGF (BD Biosciences), 20ng/ml EGF (BD Biosciences), 4 µg/ml heparin (Sigma), penicillin-streptomycin (Hyclone), and B27 (Gibco). The cells were fed 1 ml of media every 2-3 days. On day 10, all cells/spheres were collected, digested using 0.05% trypsin with 0.53mm EDTA-4Na (Invitrogen), and single cells plated as above at 2,000 cells per well to perform secondary assays. Tumorspheres were counted on day 8 of second passage.

Immunohistochemistry

Tumor array BRC711 was obtained from US Biomax (Rockville, MD, USA). Paraffin embedded sections were stained as previously described (Harrell *et al.*, 2006) following the ImmPRESS kit protocol (Vector Laboratories, Burlingame, CA). Primary antibodies included

Eya2 (1:300; Ford Lab), Six1 (1:75; Atlas Antibodies, Stockholm, Sweden), and Smad3 (5ug/ml; Invitrogen). Nuclear staining intensity in tumor cells was scored in a blinded fashion by a pathologist (PJ) on a 0-4 scale with 4 corresponding to most intense staining.

Analysis of microarray data

Gene expression and clinical outcome data were obtained from the van de Vijver and Wang datasets (van 't Veer *et al.*, 2002; Wang *et al.*, 2005). Six1 and Eya1-4 gene expression was obtained for each tumor sample. All samples were mean-centered for each gene and divided into 2 groups: samples that express the denoted gene or gene combination above (high) or below the mean (low) and the rest of the samples that did not fit the denoted gene expressions (other). Each data set was analyzed separately. Kaplan-Meier survival curves were generated using WinStat for Excel (R. Fitch Software). Normalization was obtained from the Stanford Microarray Database (van de Vijver) and NCBI GEO (Wang: GSE2034).

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary information is available at Oncogene's website.

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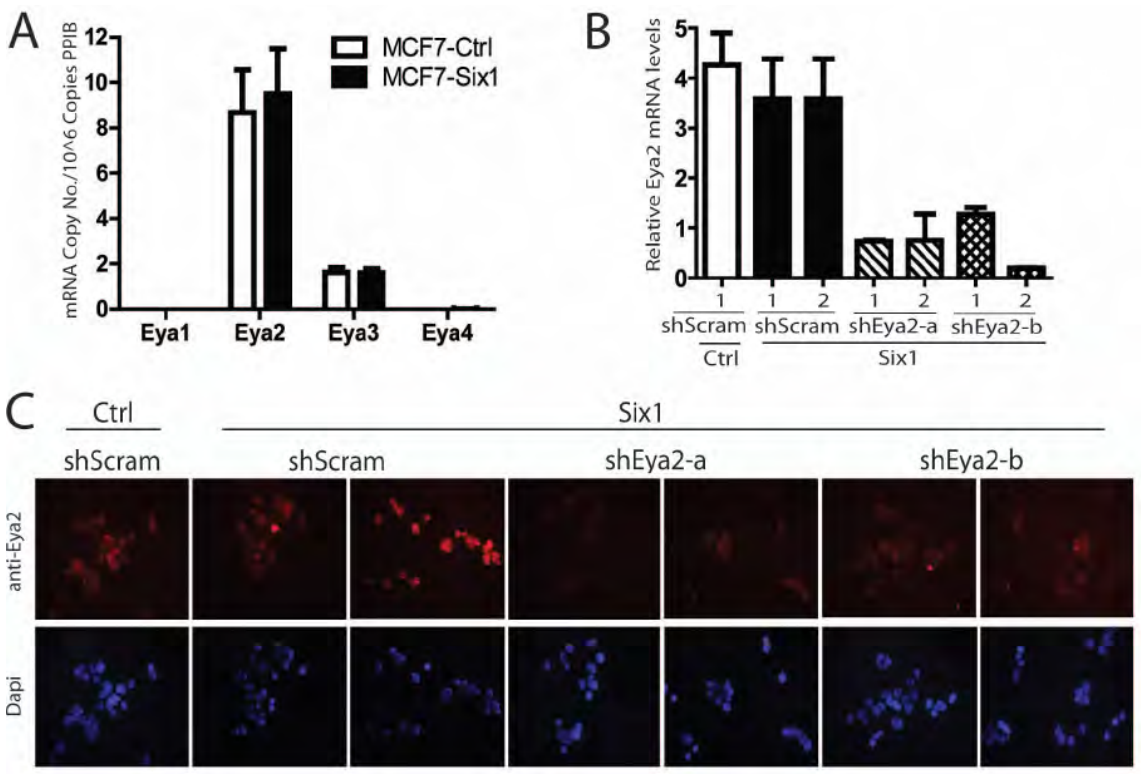


Figure 1: Eya2 is the predominant Eya in MCF7 cells and can be efficiently knocked-down in MCF7-Six1 cells. (a) Eya2 is the most abundant Eya family member in MCF7 cells. Quantitative real time PCR of Eyas1-4, indicating comparative Eya mRNA copy number in MCF7-Six1 or MCF7-Ctrl clones. Error bars represent the standard error of the mean of 3 grouped MCF7-Six1 or MCF7-Ctrl clones from a single RNA isolation. Data is a representative image of triplicate RNA isolations. (b) Real time PCR and (c) immunocytochemistry for Eya2 performed on MCF7 cells stably transfected with Eya2 shRNA or scramble shRNA, and then clonally selected. shRNA-a and shRNA-b are separate clonal isolates containing shRNA constructs targeting different regions of Eya2. Two clonal MCF7-Six1/shRNA isolates from each shRNA group were chosen for analysis. Error bars indicate the standard deviation of the mean of duplicate RNA isolations.

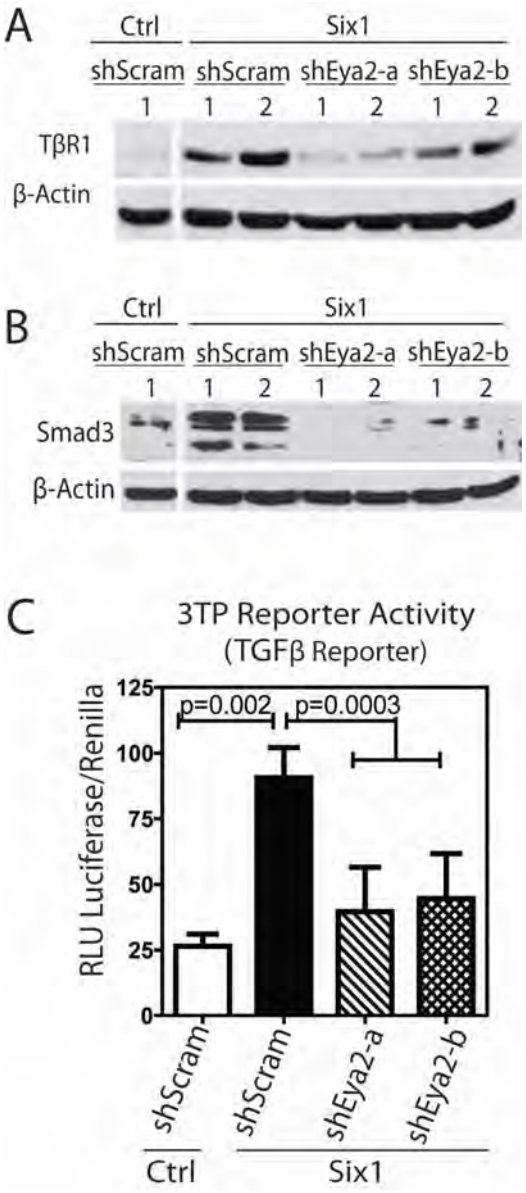


Figure 2: Eya2 knockdown in MCF7-Six1 cells reverses Six1-induced TGF-β signaling. MCF7-Six1/Eya2 shRNA clones have decreased levels of (a) TβR1 and (b) total Smad3. (c) MCF7-Six1/Eya2 shRNA clones exhibit decreased TGF-β-responsive transcription compared to MCF7-Six1/scrambled controls as observed by performing luciferase activity assays with the 3TP reporter construct. All transfections were normalized to renilla luciferase activity. Data points show the mean of two individual clones and error bars represent the standard error of the mean for 2 experiments. *P* values represent unpaired *t* test statistical analysis.

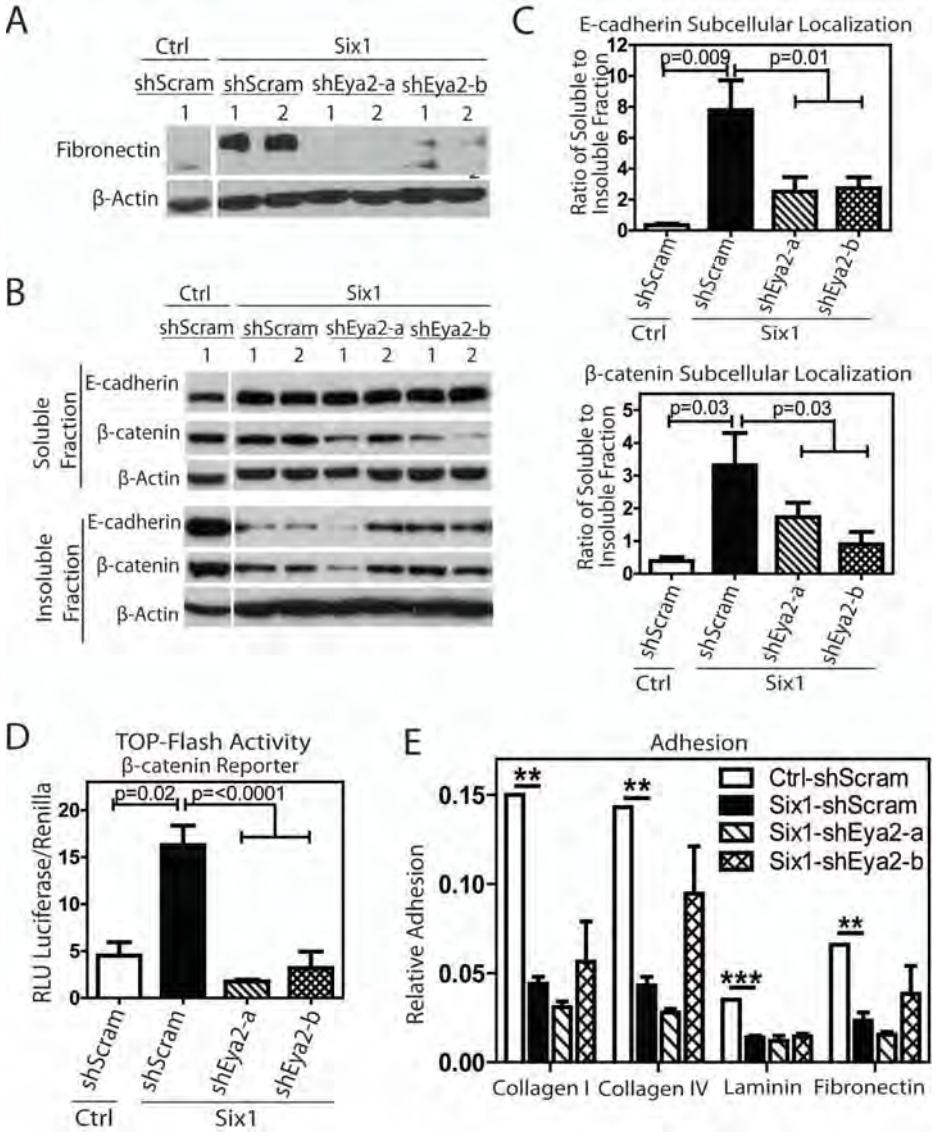


Figure 3: Eya2 knockdown partially reverses Six1-induced EMT. Eya2 knockdown in MCF7-Six1 cells reverses the ability of Six1 to (a) upregulate the mesenchymal marker Fibronectin and (b & c) to relocalize E-cadherin and β-catenin from the insoluble (membranous) fraction to the soluble (cytoplasmic) fraction as shown by western blot and quantitation following cell fractionation. (d) MCF7-Six1/Eya2 shRNA clones have decreased β-catenin responsive transcription compared to Six1 scrambled controls. β-catenin transcriptional activity was measured using the TOP-flash luciferase reporter construct normalized to renilla luciferase activity. Data points for fractionation and reporter activity show the mean of two individual clones and error bars represent the standard error of the mean for 2 experiments. *P* values represent unpaired *t* test statistical analysis. (e) Eya2 knockdown does not reverse the Six1 induced decrease in cell-matrix adhesion. Relative adherence measured by crystal violet staining.

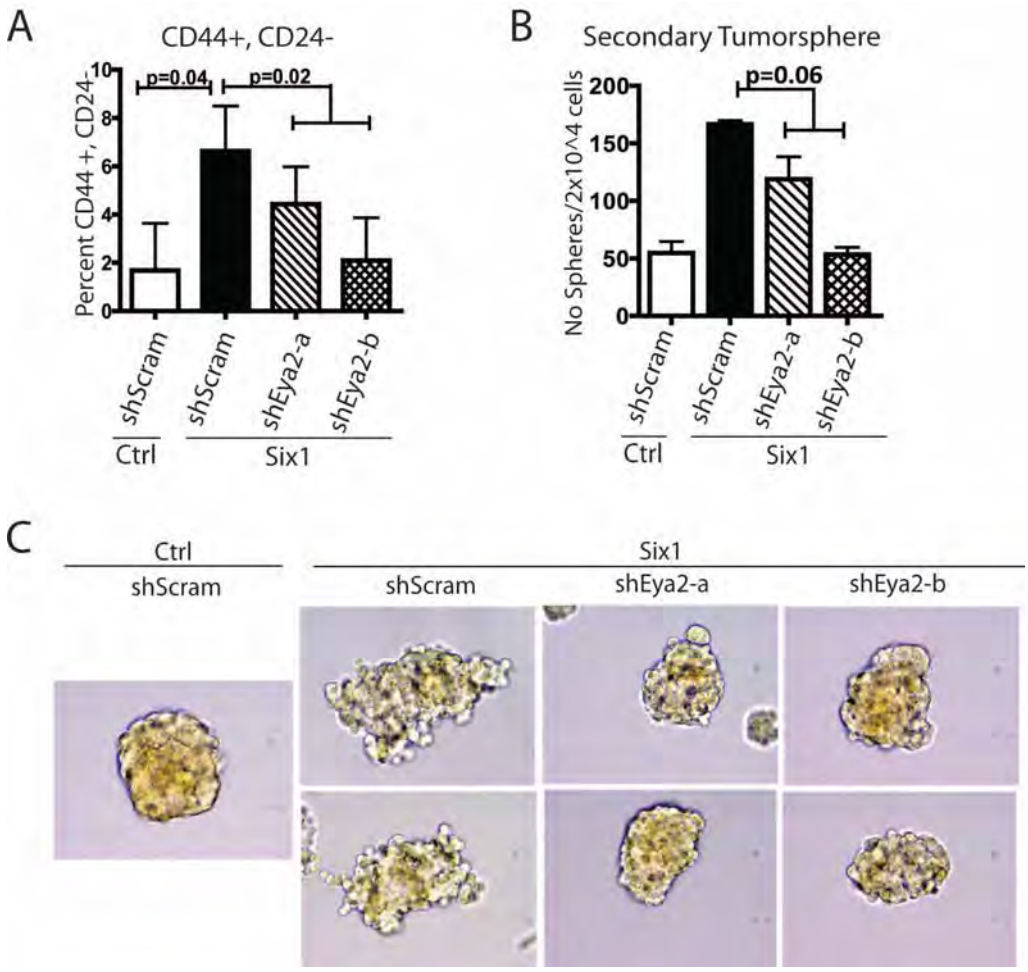


Figure 4: Eya2 knockdown reverses the Six1-induced increase in cancer stem cell characteristics. (a) Flow cytometric analysis demonstrates loss of CD44+/CD24- cancer stem cells with knockdown of Eya2. (b) Secondary tumorsphere assays, a measurement of self renewal capacity, demonstrate decreased tumorsphere formation and (c) re-gained spherical shape with knockdown of Eya2. Antibodies used to perform flow cytometry include CD24 and CD44, markers found on human epithelial stem cells. Figure is a representative image of two experiments. Data points show the mean of two individual clones and error bars represent the standard error of the mean. *P* values represent unpaired *t* test statistical analysis. Original magnification, x200.

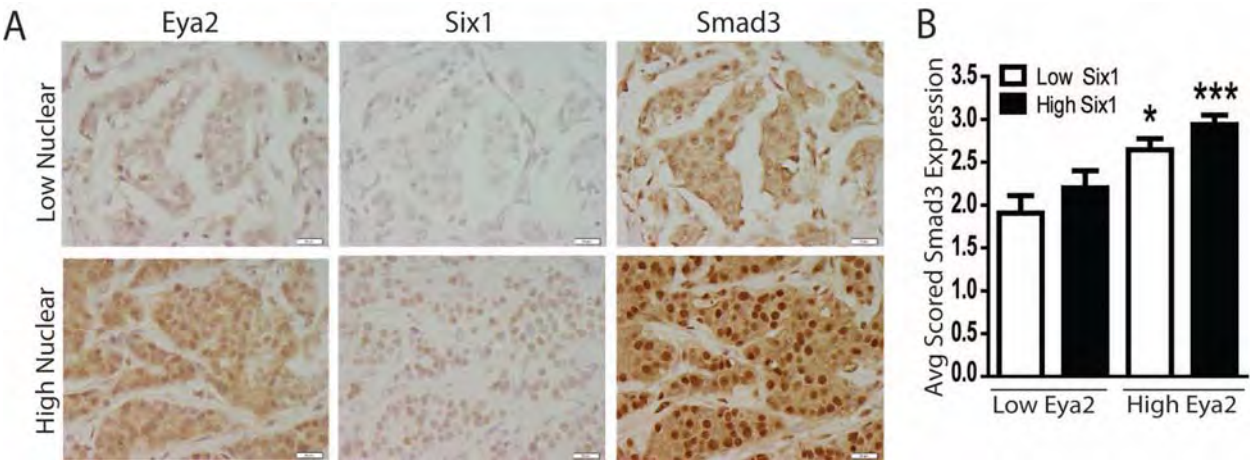


Figure 5: Co-expression of Eya2 and Six1 correlates with activated TGF-β signaling in human breast cancer. **(a)** Representative images of human breast cancer tissue arrays stained with anti-Six1, anti-Eya2, and anti-Smad3 antibodies show that low Six1 and low Eya2 correlate with little nuclear Smad3, whereas tumors that express both high Six1 and Eya2 show high levels of nuclear Smad3. (b) Quantitation of Smad3 staining. Staining of tissues was scored on a scale from 0-4 for each antibody. Each sample was categorized as having above (high) or below (low) the mean Eya2 and/or Six1 staining and values were plotted against the average Smad3 scores within the group. Statistical analysis performed using Anova. Original magnification, x400. Scale bars: 20um.

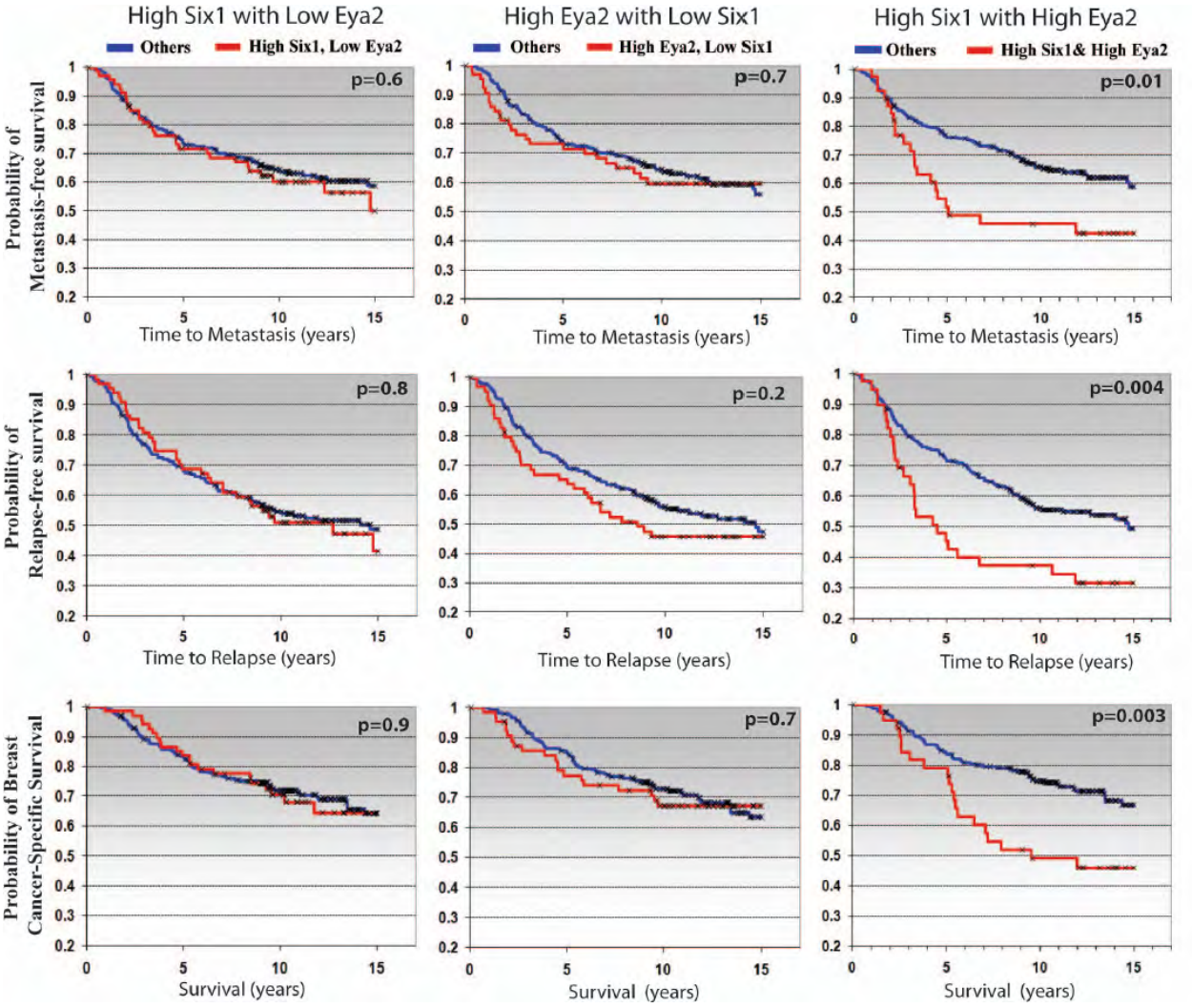


Figure 6: Coordinate high Six1 and Eya2 expression are required to predict poor prognosis in human breast cancer. In a gene expression dataset of 295 women with early-stage invasive breast carcinoma (van 't Veer *et al.*, 2002), patient samples expressing high Six1 in the absence of high Eya2, and high Eya2 in the absence of high Six1, do not correlate with reduced time to metastasis, reduced time to relapse, and shortened breast cancer-specific survival while high Six1 and high Eya2 together in the same patient sample significantly correlates with shortened time to relapse and to metastasis, and with shortened breast cancer specific survival. The mean value for Six1 and/or Eya2 expression was used to divide the samples into high (above the mean) and low (below the mean) Six1 and Eya2 expression. P-values were calculated by log-rank analysis.

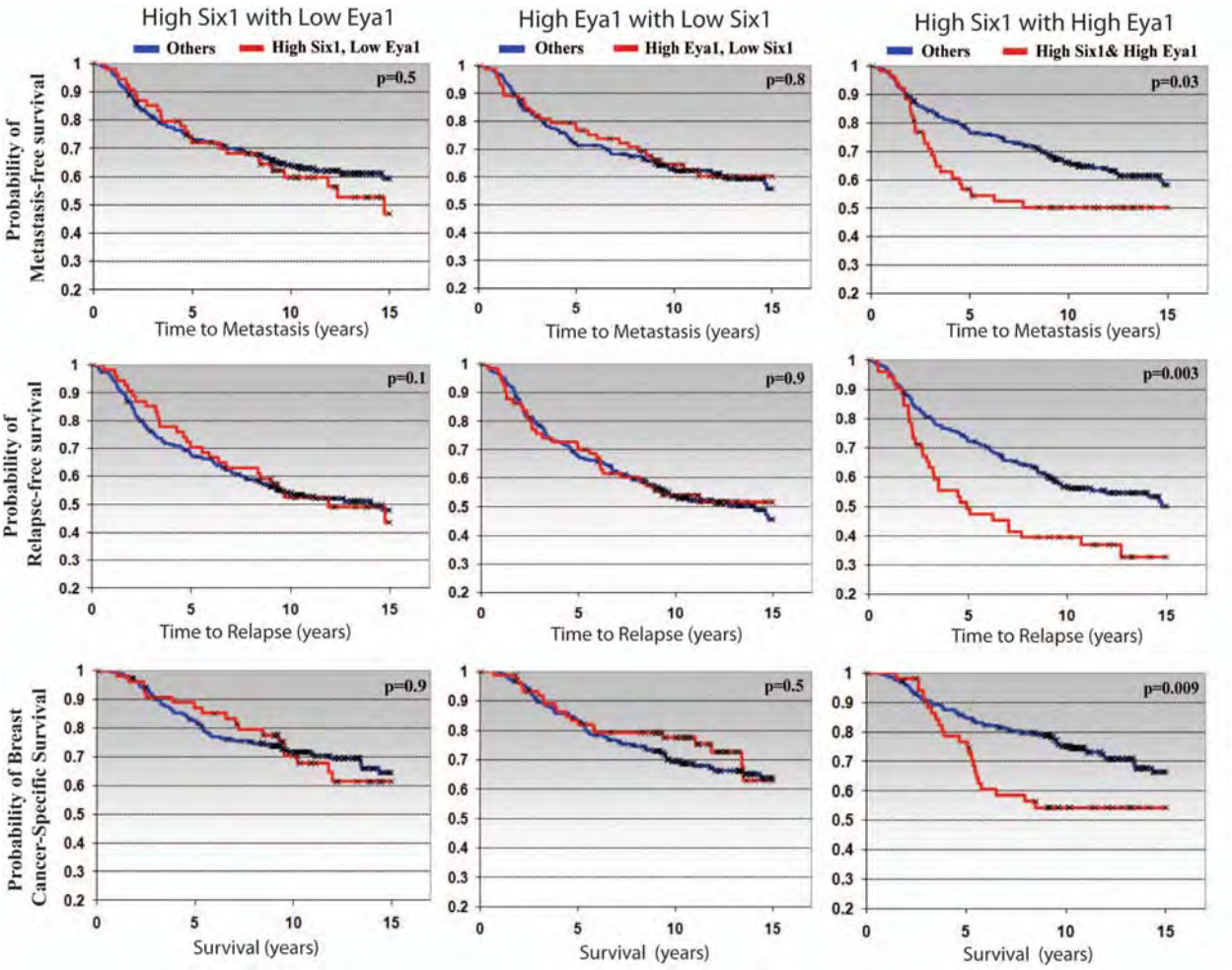
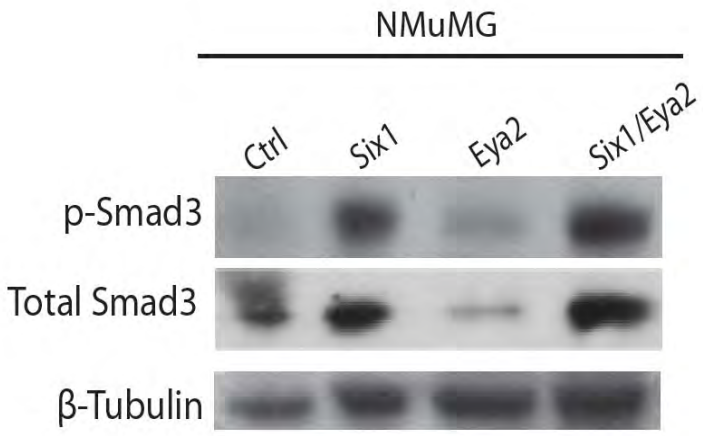
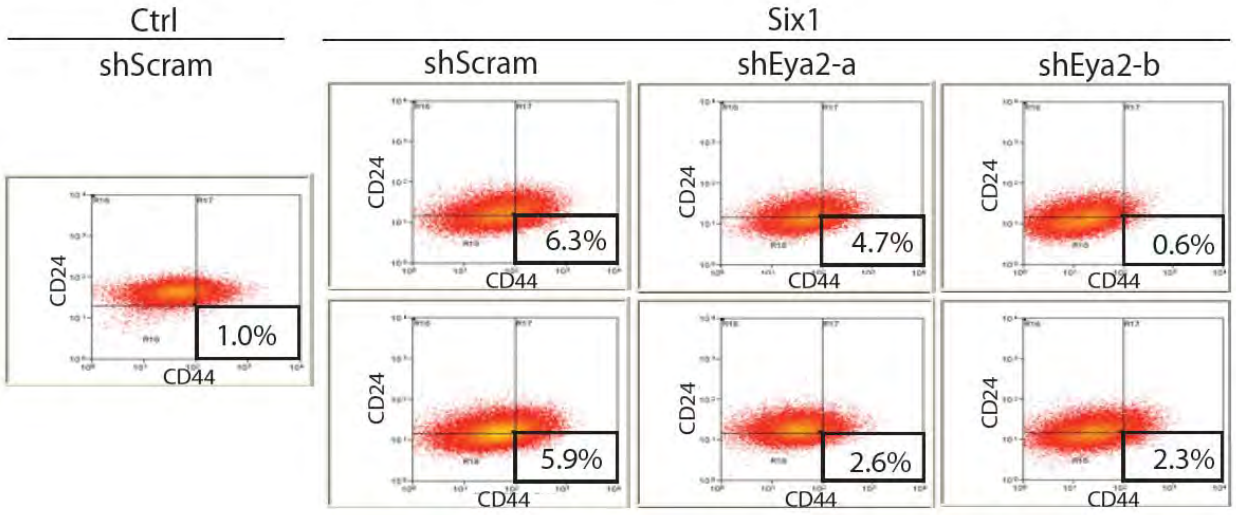


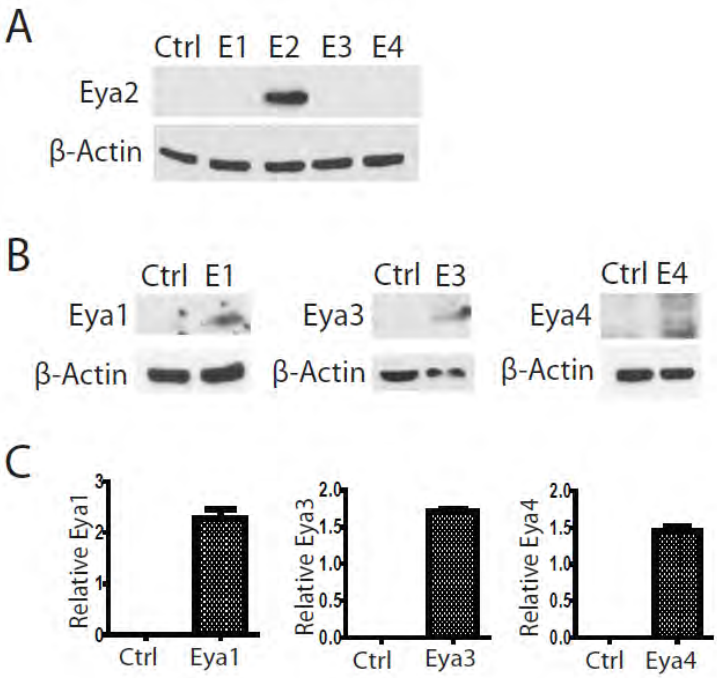
Figure 7: Coordinate high Six1 and Eya1 expression are required to predict poor prognosis in human breast cancer. In a gene expression dataset of 295 women with early-stage invasive breast carcinoma (van 't Veer *et al.*, 2002), patient samples expressing high Six1 in the absence of high Eya1, and high Eya1 in the absence of high Six1, do NOT correlate with reduced time to metastasis, time to relapse and shortened breast cancer specific survival while high Six1 and high Eya1 together in the same patient sample significantly correlates with the aforementioned prognostic indicators. The mean value for Six1 and/or Eya1 expression was used to divide the samples into high (above the mean) and low (below the mean) Six1 and Eya1 expression. P-values were calculated by log-rank analysis.



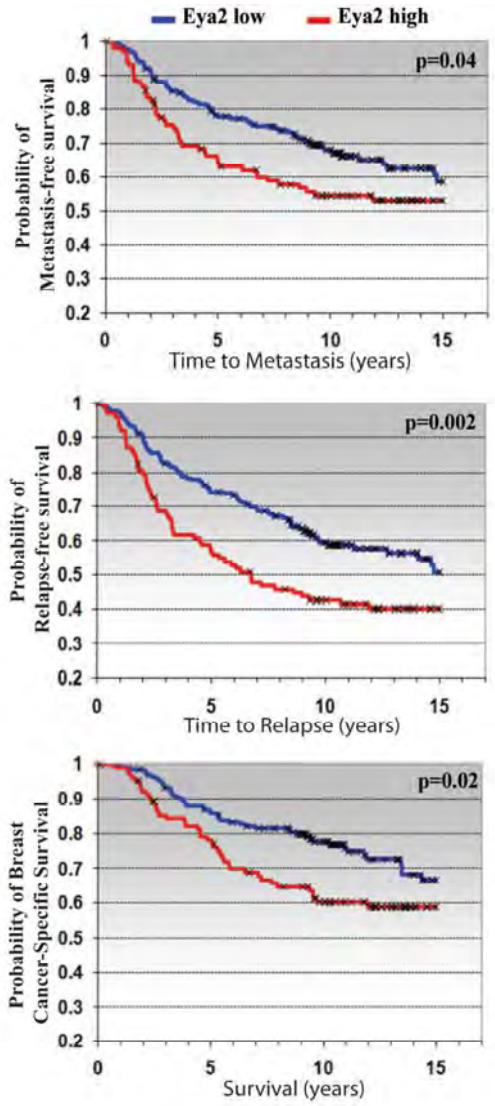
Supplemental Figure 1: Co-expression of Six1 and Eya2 increases total and p-Smad3 levels. Western blot analysis on whole cell lysates of NMuMG cells expressing Six1 and/or Eya2 demonstrates that Six1 and Eya2 together increase total and p-Smad3 levels above that observed with individual expression, suggesting cooperation to increase TGF-β signaling activation.



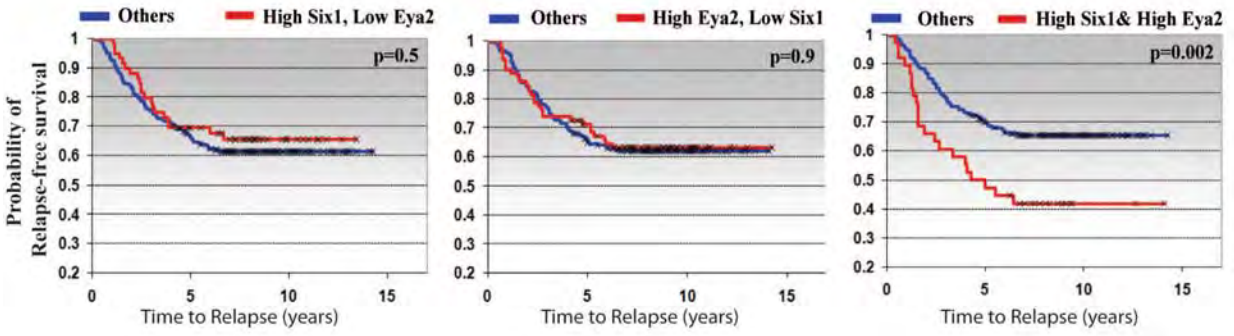
Supplemental Figure 2: Eya2 knockdown reverses the Six1-induced increase in the CD44+/CD24- cell population. Flow cytometric analysis demonstrates loss of the CD44+/CD24- cancer stem cell population with knockdown of Eya2 in MCF7-Six1 cells. Individual representation of duplicate experiments combined in Figure 4.



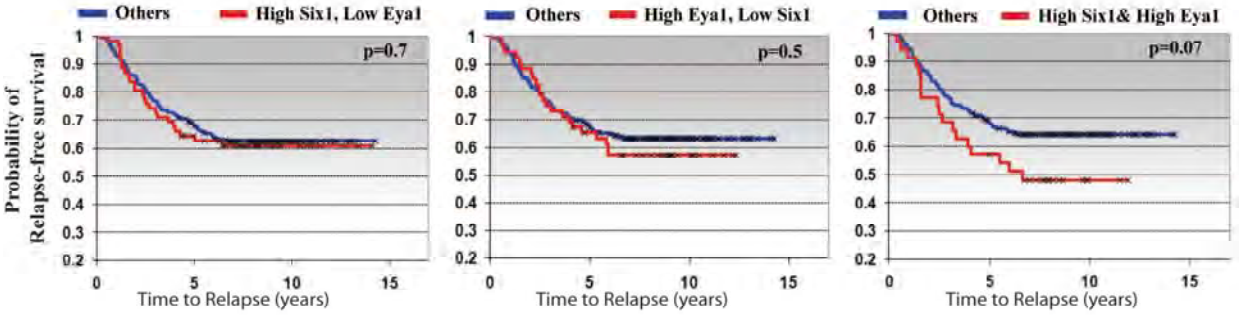
Supplemental Figure 3: The Eya2 antibody specifically recognizes Eya2. Western blot analysis on whole cell lysates from MCF7 cells transfected with each Eya using (a) the Eya2 or (b) Eya1, 3, or 4 antibodies. (c) For additional transfection control, Eya1, 3, and 4 expressions were also checked using real-time PCR.



Supplemental Figure 4: High Eya2 expression correlates with poor prognosis in human breast cancers. In the van de Vijver early-stage invasive breast cancer microarray dataset (van't Veer *et al.*, 2002), expression of high Eya2 (above mean) correlates with poor prognosis to a comparative level published for Six1(Micalizzi *et al.*, 2009). P-values analyzed by log-rank.



Supplemental Figure 5: In the Wang breast cancer dataset, coordinate high Six1 and Eya2 expression predict poor prognosis. In lymph-node negative patients (Wang *et al.*, 2005), only patient samples expressing high Six1 and high Eya2 together (above the mean) significantly correlate with reduced time to relapse. P-values analyzed by log-rank.



Supplemental Figure 6: In the Wang breast cancer dataset, coordinate high Six1 and Eya1 predict poor prognosis. In lymph-node negative patients (Wang *et al.*, 2005), only patient samples expressing high Six1 and high Eya1 together (above the mean) significantly correlate with reduced time to relapse. P-values analyzed by log-rank.

Gene	Flourescence	Probe 5'-3'	Forward Primer 5'-3'	Reverse Primer 5'-3'
Eya1	TaqMan	AGCCTGGCTGCAGTTGAGGGCC	TGGTTGTCTGCTTGGTCCAG	AGTCGGTCAGGGCTTCAATTT
Eya2	TaqMan	TAAACCTCATCAACTCCCGGCCCAA	GACCCACTCCCTGAAGGCA	GGTGACCAGCACATTGACACA
Eya3	TaqMan	AGAACGGGTATTTCTGTGGGACTTGGATG	TGATGCCACTTCTTCCCAAGA	AAGTGAGTGGAAGATGATGATGGTT
Eya4	TaqMan	CAGAAGTATGGCAAGGATCCCCCCAT	TGCTCACCGGGTCTTATGC	CGGAGTCCAAGGGTTACAGC
PPIB	TaqMan	ABI : Assays On Demand - Hs01018503		

Supplemental Figure 7: Real-time PCR probes and primers. Real time PCR was performed using TaqMan fluorescence probes. PPIB endogenous control primers and probe were acquired from Assays On Demand (ABI).